REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	9/26/01	Manuscript

4. TITLE AND SUBTITLE
Detection of Ciprofloxacin Resistant Yersinia pestis using Fluorogenic PCR Measured in the Lightcycler

5. FUNDING NUMBERS
PE 9G8D

6. AUTHOR(S)
Luther E. Lindler*, Wei Fan and Nazma Jahan

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Bacterial Diseases, Div. of Communicable Disease and Immunology, WRAIR, Silver Spring, MD 20910

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Medical Research and Materiel Command

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
To be published in the Journal of Clinical Microbiology, 2001, Volume 39, pages 3649-3655.

12a. DISTRIBUTION / AVAILABILITY STATEMENT

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

We have developed a fluorescence resonance energy transfer-based (FRET) assay to detect ciprofloxacin resistant (Cpr) mutants of the biothreat agent Yersinia pestis. We selected spontaneous mutants of the attenuated Y. pestis KIM 5 strain that were resistant to at least 1 mg/ml Cp. DNA sequencing of gyrA encoded by sixty-five of these mutants revealed that all isolates contained one of four different point mutations within the quinolone resistance-determining region of gyrA. We developed a FRET assay that detected all of these mutations using a single pair of fluorescent probes with sequences complementary to the wild type Y. pestis gyrA sequence. Melting peak analysis revealed that the probe-PCR product hybrid was less stable when amplification occurred from any of the four mutant templates. This instability resulted in the PCR product obtained from the Cpr Y. pestis strains displaying a 4-11OC shift in probe melting temperature. Following optimization of the reaction conditions we were able to detect approximately 10 pg of purified wild type template DNA or the presence of approximately 4 CFU of Y. pestis KIM 5 wild type or Cpr mutants in crude lysates. Taken together, our results demonstrate the utility of FRET-based assays to detect Cpr mutants of Y. pestis that is both sensitive and rapid.

14. SUBJECT TERMS
Antibiotic resistance, Biological warfare, Diagnostics

15. NUMBER OF PAGES
7

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2001, p. 3649–3655
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.10.3649–3655.2001

Vol. 39, No. 10

Detection of Ciprofloxacin-Resistant *Yersinia pestis* by Fluorogenic PCR Using the LightCycler

LUTHER E. LINDLER,* WEI FAN, AND NAZMA JAHAN

Department of Bacterial Diseases, Division of Communicable Diseases and Immunology, Walter Reed Army Institute of Research, Silver Spring, Maryland 20910

Received 7 May 2001/Returned for modification 9 July 2001/Accepted 26 July 2001

We have developed a fluorescence resonance energy transfer (FRET)-based assay to detect ciprofloxacin resistant (Cp^r) mutants of the biothreat agent *Yersinia pestis*. We selected spontaneous mutants of the attenuated *Y. pestis* KIM 5 strain that were resistant to a ciprofloxacin (CIP) concentration of at least 1 μg/ml. DNA sequencing of *gyrA* encoded by 65 of these mutants revealed that all isolates contained one of four different point mutations within the quinolone resistance-determining region of *gyrA*. We developed a FRET-based assay that detected all of these mutations by using a single pair of fluorescent probes with sequences complementary to the wild-type *Y. pestis gyrA* sequence. Melting peak analysis revealed that the probe-PCR product hybrid was less stable when amplification occurred from any of the four mutant templates. This instability resulted in the PCR product obtained from the Cp^r *Y. pestis* strains displaying a 4 to 11°C shift in probe melting temperature. Following optimization of the reaction conditions, we were able to detect approximately 10 pg of purified wild-type template DNA or the presence of approximately 4 CFU of wild-type *Y. pestis* KIM 5 or Cp^r mutants in crude lysates. Taken together, our results demonstrate the utility of FRET-based assays for detection of Cp^r mutants of *Y. pestis*. This method is both sensitive and rapid.

Resistance to antibiotics has become a major concern for the medical community over the past several years (13, 14, 16). Many organisms have become resistant to the common "drug of choice" used to treat the disease. A few examples are methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci (18), and multiple-drug-resistant *Mycobacterium tuberculosis* (19), as well as organisms causing many enteric diseases. One of the current antibiotics that is effective in treating bacterial infectious diseases is ciprofloxacin (CIP), a fluorinated quinolone that blocks DNA replication through inhibition of gyrase activity (2, 24). Resistance to CIP does occur and is usually mediated by point mutations in DNA gyrases or, less commonly, through membrane alterations that reduce drug entry into the bacteria (28).

A critical piece of information necessary for the treatment of any bacterial disease is the antibiotic sensitivity profile of the infectious agent. Classically the sensitivity profile has been determined by growth of the organism in the presence of the antibiotic either in agar diffusion assays or by incubation of the organism in various concentrations of the drug for determination of the MIC. Both of these methods depend on growth of the bacterium after its initial isolation and are therefore time-consuming. DNA probe-based detection of antibiotic resistance offers the potential for increased speed. Among DNA-based techniques, PCR offers the best opportunity for speed, sensitivity, and specificity.

Recently it has become possible to couple PCR with real-time detection of the amplification product by use of fluorescent probes, thus eliminating the necessity to analyze the reaction product by gel electrophoresis. Fluorescence resonance energy transfer (FRET) is one of the available chemistries that can be used to detect the PCR product in these reactions. Roche Diagnostics has adopted this chemistry for its "Hybridization Probes" technology (5). Two DNA probes are used to bind to the amplification product when FRET chemistry is used to specifically detect the amplification product. The two light-activated molecules are positioned in close proximity at the 3′ and 5′ termini of the probes such that fluorescence increases as more PCR product accumulates and the two labeled probes bind next to each other.

The increase in antibiotic resistance coupled with the threat of modification of agents of biological warfare have prompted us to develop a hybridization probe assay for the detection of CIP resistance (Cp^r) in *Yersinia pestis*. We chose *Y. pestis* as a model for our initial development of a Cp^r assay for three reasons. First, it is the etiologic agent of a disease that has high potential for use as a biological terrorism or biological warfare agent (27). Second, it is likely that any biological used as a weapon would be made antibiotic resistant. Third, *Y. pestis* is relatively slow growing, and therefore the increased speed of DNA-based antibiotic resistance detection would improve our ability to properly treat infected individuals. Although our development of the assay used *Y. pestis* as a model, the method should be broadly applicable to the detection of antibiotic resistance encoded by point mutations in other organisms.

MATERIALS AND METHODS

Cultivation of bacteria, isolation of Cp^r mutants, and antibiotic sensitivity testing. The bacterium used in this study was an avirulent, pigmentation-negative (*pgm*-negative) mutant of *Y. pestis* KIM 5 and was obtained from Susan Straley, Department of Microbiology and Immunology, University of Kentucky, Lexington, Ky. Bacteria were grown on brain heart infusion agar (BHI; Difco Laboratories, Detroit, Mich.) plates for 48 h at 30°C or overnight in BHI broth at 30°C with aeration. After cultivation, the bacteria were collected, washed twice with phosphate-buffered saline (150 mM NaCl, 1.7 mM KH_2PO_4, 5 mM Na_2PO_4 [pH 7.4]) and suspended to yield a concentration of 10^{11} CFU/ml. For selection of

* Corresponding author. Mailing address: Department of Bacterial Diseases, WRAIR, 503 Robert Grant Ave., Silver Spring, MD 20910. Phone: (301)-319–9388. Fax: (301) 319-9123. E-mail: Luther.Lindler@na.amedd.army.mil.

Cp^r mutants, a 100-μl sample of washed bacteria was plated on BHI agar plates containing 1 to 10 μg of CIP (Bayer, Leverkusen, Germany)/ml. Cp^r mutants appeared after 48 to 72 h of incubation at 30°C. Colonies were purified by streaking on fresh BHI agar plates containing the appropriate concentration of CIP. The MIC for CIP was determined by using E Test (AB Biodisk, Piscataway, N.J.) antibiotic strips containing a continuous gradient of CIP. The E Test was performed by spreading 100 μl of bacterial culture onto BHI agar plates without antibiotic. After 48 h of incubation at 30°C, the MIC was read according to the manufacturer's instructions.

DNA isolation, gene amplification, and sequencing. Total-cell DNA was extracted from 5-ml cultures of *Y. pestis* or Cp^r mutants with the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, Minn.). Initially primers to amplify the quinolone resistance-determining region (QRDR) (30) of *gyrA* were selected from the published *Escherichia coli* sequence (GenBank accession number AE000312) (3) using Primerselect software (Lasergene, Madison, Wis.). PCR amplification primers gyrA51 and gyrA31 were ATGAGCGACCTTGCGA GAG and TGTTCCATCAGCCCTTCAATG, respectively. We used PCR and direct DNA sequencing to identify *Y. pestis gyrA* mutations. PCR was carried out with AmpliTaq Gold (Perkin-Elmer, Foster City, Calif.) according to the manufacturer's directions. Initial activation of AmpliTaq Gold and denaturation of template DNA were carried out at 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension step of 72°C for 7 min. Amplification products were examined for size and purity by fractionation of a 10-μl sample on 1.5% agarose gels or 10% polyacrylamide gels (Novex Corporation, San Diego, Calif.). PCR products were purified for automated DNA sequencing with a QIAquick PCR Purification Kit (Qiagen, Valencia, Calif.). Nucleotide sequence determination was performed by the dideoxy chain-termination method using the Applied Biosystems International (ABI, Foster City, Calif.) PRISM dichloRhodamine Dye Terminator Sequencing Kit with AmpliTaq DNA polymerase. DNA sequencing reactions were analyzed on an ABI 377XL automated DNA sequencher. Sequence data were edited and assembled into contiguous sequences using the Sequencher program (Gene Codes, Ann Arbor, Mich.). The *Y. pestis* KIM 5 wild-type *gyrA* nucleotide sequence was determined using the gyrA51 and gyrA31 primers used in the initial PCR. The DNA sequences of the mutant *gyrA* genes were determined using oligonucleotide primers gyrA51seq and gyrA31seq, which had the sequences AAATAACACC GGTCAACATC and ATATAAGCCAGACAGCCATCA, respectively. Template DNA for sequencing of the *Y. pestis gyrA* mutants was prepared by using 2.5-μl boiled overnight bacterial culture as the template in a 50-μl PCR mixture with gyrA51 and gyrA31 as the primers. The purified PCR product obtained from the mutants was sequenced as described above for wild-type *Y. pestis gyrA*.

The QRDR regions of *Y. pestis gyrB* and *parC* were amplified using oligonucleotide primer pairs GyrB51 (TCGAATTCTTATGACTCCTCCAG)–GyrB31 (CAATACCGTCTTTTTCAGTGGAG) and ParC53 (GACCGTGCGTTGCC GTTTATTGG)–ParC39 (TTGGCTAAGTGGCGGAGTTTC). These primers were selected by using the *E. coli* GyrB (GenBank accession number P06982) and ParC (P20083) protein sequences (3) to search the *Y. pestis* CO92 genome database at http://www.sanger.ac.uk/Projects/Y_pestis/blast_server.shtml (1). Contiguous sequences that could encode proteins 78% identical to GyrB and 80% identical to ParC over the entire length of the *E. coli* proteins were found in the *Y. pestis* CO92 genome database. The contiguous DNA sequences from the genome database were used to obtain the potential coding regions for *Y. pestis* KIM 5 GyrB and ParC.

FRET probe detection of Cp^r mutants. *Y. pestis* Cp^r mutants were detected using hybridization probe (FRET) reactions with the LightCycler (Roche Diagnostics, Mannheim, Germany). The primers for hybridization probe reactions were LC3 (GATTATGCGATGTCCGTTATTGTC) and LC4 (GAAGTTACC CTGCCCATCCAC). Primers LC3 and LC4 were designed using Primerselect (Lasergene). Sequence-specific hybridization probes were probe 1 (GCATGGT GACAGCGCGGTC-fluorescein) and probe 2 (Lightcycler Red 640-ACGACA CTATCGTGCGTATGGCCCA-PO_4). The probes were chosen using Primerselect (Lasergene) software according to the guidelines suggested by Roche Diagnostics. Operon Technologies, Inc. (Alameda, Calif.), or Synthegen LLC (Houston, Tex.) synthesized the fluorescent-labeled FRET probes. Mutant sequence detection was performed with primers LC3 and LC4 plus probe 1 and probe 2 and the Roche Diagnostics DNA Master Hybridization Probes kit. The optimized reaction mixture contained template DNA in 2 μl of water, 5 mM $MgCl_2$, 1 μM primers LC3 and LC4, 0.4 μM (each) probe1 and probe2, and 1× LightCycler DNA Master Hybridization Probes buffer, according to the manufacturer's directions (Roche Diagnostics). Crude lysates of Cp^r mutants were analyzed by a simple growth and boiling procedure. A 2-μl portion of the clarified supernatant obtained from crude boiled lysates was used as a template in the hybridization probe Cp^r detection assay as described above. All PCRs were performed on a LightCycler (Roche Diagnostics) using channel F2 (640 nm). The *Y. pestis gyrA* target sequences were amplified by a single incubation at 95°C for 1 min, followed by 40 cycles of 95°C for 0 s, 58°C for 15 s, and 72°C for 15 s. The melting curve for the annealing of the PCR product with the FRET probes was determined by monitoring the fluorescence of channel F2 from 40 to 95°C with a temperature transition rate of 0.1°C per s. Lightcycler amplification results were verified by electrophoresis of 10 μl of the PCR product on 1.5% agarose gels. Data were analyzed with LightCycler software, version 3.1, according to the manufacturer's instructions.

Nucleotide sequence accession numbers. The DNA sequences of wild-type *Y. pestis* KIM 5 and of the four examples of *Y. pestis* KIM 5 Cp^r *gyrA* point mutants have been deposited in GenBank and assigned accession numbers AF217736 through AF217740. The *Y. pestis gyrB* and *parC* sequences have been given GenBank accession numbers AF221694 and AF221695, respectively. To promote ease of analysis and comparison with previous studies, we have adopted the convention of using the *E. coli* GyrA (GenBank accession number P09097) numbering throughout this paper when referring to specific amino acid residues.

RESULTS

Isolation of Cp^r *Y. pestis* mutants. We performed 11 independent experiments to isolate Cp^r mutants. The average mutation frequency observed for all of these experiments was 2.6×10^{-10}. These mutant isolations resulted in 682 Cp^r *Y. pestis* KIM 5 strains. We chose 65 mutants at random for further single-colony isolation and characterization. We performed initial experiments to determine the influence of plating cell density on the CIP MIC observed by using E Test antibiotic sensitivity strips. Inoculation of BHI agar plates with approximately 10^7 or 10^8 cells produced MICs that were similar to each other. However, inoculation of BHI agar plates for the E Test with cell concentrations above or below 10^7 or 10^8 CFU produced significantly higher or lower observed MICs, respectively. Accordingly, we chose to plate *Y. pestis* KIM 5 at 10^7 CFU per plate in order to determine the observed MIC for CIP in all later experiments. Our observed CIP MIC for the wild-type *Y. pestis* strain KIM 5 was 0.026 μg/ml. For all the Cp^r mutants, MICs at least 40 times greater than that for the wild type, i.e., between 1.1 and 4.6 μg/ml, were observed (Fig. 1B).

Sequencing of wild-type *Y. pestis* and Cp^r mutants. We obtained a single, approximately 630-bp fragment of DNA from the *Y. pestis* KIM 5 genome using oligonucleotide primers gyrA51 and gyrA31 in PCRs. After determination of the sequence of this fragment on both strands of DNA, a BLASTN (1) search of GenBank (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) revealed that our sequence had a high degree of homology (probability value, $1e^{-137}$) with *Serratia marcescens gyrA* (GenBank accession number AF052260) (26). The final 492-bp *Y. pestis* KIM 5 *gyrA* sequence was 88% identical to *S. marcescens gyrA* at the nucleotide level, suggesting that it was the plague homologue of the gyrase A gene. Further evidence was obtained by performing protein searches of the nonredundant GenBank database. The protein database search revealed that the putative protein product translated from our DNA sequence was 96% identical over a 164-amino-acid overlap with *S. marcescens* GyrA (AAC68576) (26) as well as many other *E. coli* GyrA homologues. Our analysis of the predicted *Y. pestis* KIM 5 GyrA sequence obtained from the PCR product using Megalign (Lasergene) revealed that the nucleotide sequence changes encoded six conservative amino acid substitutions compared to *S. marcescens* GyrA. Our results revealed that the *Y. pestis* KIM 5 GyrA sequence within the QRDR was

A.

M1	ACCACCCGCATG**A**TGACAGCGCGGTCT	Gly to Asp at pos 81
M2	ACCACCCGCAT**T**GTGACAGCGCGGTCT	Gly to Cys at pos 81
M3	ACCACCCGCATGGTGACA**T**CGCGGTCT	Ser to Ile at pos 83
M4	ACCACCCGCATGGTGACAG**A**GCGGTCT	Ser to Arg at pos 83
WT	ACCACCCGCATGGTGACAGCGCGGTCT	Wild Type

B.

E. coli 67 ARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLRYMLVDGQ 106

		Number Identified/Total	MIC in μg/ml
Y. pestis M1	D	37/65	4.10 (1.37)
M2	C	6/65	1.33 (0.44)
M3	I	11/65	1.31 (0.39)
M4	R	11/65	4.35 (1.09)

FIG. 1. Nucleotide sequence and protein changes in *Y. pestis* Cp^r mutants. (A) DNA sequences of wild-type (WT) *Y. pestis gyrA* and the four point mutants, identified as M1 through M4, corresponding to their designations in the text. Underlined nucleotides in the WT sequence denote the FRET assay probe 1. Nucleotide substitutions in mutants M1 through M4 are boldfaced and underlined. Amino acid substitutions and the position relative to *E. coli* GyrA (3) are given on the right. (B) Amino acid changes, isolation frequency, and CIP MIC for the various Cp^r mutants. Amino acid numbering (67 to 106) is relative to the *E. coli* GyrA sequence (3) and is indicated to the left and right of the sequence. Boldfaced, underlined letters indicate amino acid changes in the QRDR of *E. coli* GyrA that have been shown to result in Cp^r. MICs for the *Y. pestis* mutants determined by duplicate E Tests in five independent experiments are shown as averages with standard deviations in parentheses. Amino acid changes for mutants M1 through M4 are shown as boldfaced letters below the corresponding positions in the *E. coli* QRDR.

identical to that of *E. coli.* Accordingly, we designated our sequence *Y. pestis gyrA*.

We amplified the DNA encoding the QRDR from all of the Cp^r *Y. pestis* mutants we isolated using the gyrA51–gyrA31 primer pair. The DNA sequences for each of these mutants were determined on both strands using oligonucleotide primers gyrA51seq and gyrA51seq. Each of the mutants revealed a single-nucleotide change in the QRDR of *gyrA*, and these substitutions belonged to one of four groups (Fig. 1A). The largest group of mutants isolated had a transition of guanine to adenine such that Gly-81 (relative to *E. coli* numbering, GenBank accession number P09097) was changed to Asp in that position (mutant M1 in Fig. 1). The smallest group of mutants had a guanine altered to a thymine such that Gly-81 was changed to Cys (mutant M2 in Fig. 1). The final two groups of mutants were found in equal numbers as shown in Fig. 1. We identified mutations in *Y. pestis gyrA* that were guanine-to-thymine transversions. These mutants coded for an Ile residue at position 83 instead of the Ser residue in the wild-type protein sequence (mutant M3 in Fig. 1). The last group of mutants had a transversion of cytosine to adenine such that amino acid Ser-83 was changed to Arg (mutant M4 in Fig. 1). The position of the amino acid substitution encoded by the mutant did not correlate with the level of Cp^r, since we observed MICs of approximately 1 and 4 μg/ml for strains with mutations at either codon 81 or codon 83 within *gyrA* (Fig. 1B).

Since other gene products such as GyrB (8, 12, 31) and ParC (6, 11, 20) have been shown to be involved in resistance to CIP, we amplified the QRDRs of these genes from a randomly selected group of our Cp^r mutants and compared the DNA sequences for both *Y. pestis* KIM 5 *gyrB* and *parC*. The DNA sequences of the wild type *Y. pestis* KIM 5 *gyrB* and *parC* loci were determined as described in Materials and Methods. In order to confirm that the genes we amplified did encode the expected proteins, our nucleotide sequences were translated into predicted proteins and used to search the GenBank protein database. The protein product predicted by translation of the wild-type *Y. pestis gyrB* sequence was highly homologus (probability value, $1e^{-102}$) with *E. coli* GyrB (AAC76722) (3). The entire protein sequence predicted by translation of *Y. pestis* KIM 5 *parC* was 86% identical with the *E. coli* ParC sequence (P20082) (3), amino acids 12 through 394. Accordingly, the putative function of the proteins encoded by our PCR product obtained after *Y. pestis gyrB* and *parC* amplification was confirmed by protein homologies in the database. The DNA sequence of *gyrB* carried by 12 of our 65 *Y. pestis* Cp^r mutants was determined from randomly selected strains regardless of observed MIC and was found to be identical with the wild-type sequence. Similarly, the *parC* sequence encoding the QRDR was determined for 36 of the 65 Cp^r mutants and was found to be identical to the wild-type DNA sequence.

Amplification and detection of *gyrA* in the LightCycler. We designed PCR primers and hybridization probes for detection of *Y. pestis* KIM 5 *gyrA* as shown in Fig. 2. Oligonucleotide

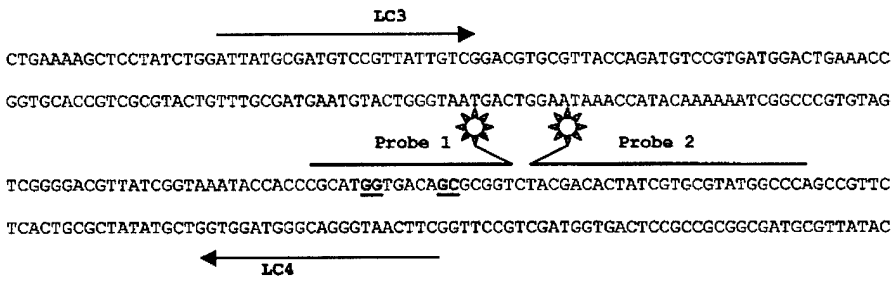

FIG. 2. Schematic representation of the hybridization probe assay for Cp^r in *Y. pestis*. PCR primers are represented by arrows above or below the *Y. pestis gyrA* sequence and are labeled LC3 and LC4. Probe 1 and probe 2 are shown between LC3 and LC4. The starbursts at the 3′ and 5′ termini of probe 1 and probe 2, respectively, indicate light reactive labels. Probe 1 is homologous with the wild-type *Y. pestis gyrA* sequence. The positions of the four point mutations described in Fig. 1A are indicated (boldfaced, underlined letters) in the wild-type DNA sequence below probe 1. The T_m of probe 1 was chosen to be less than that of probe 2 such that detection of melting of the probes from the PCR product would be dependent on the stability of probe 1 with the product.

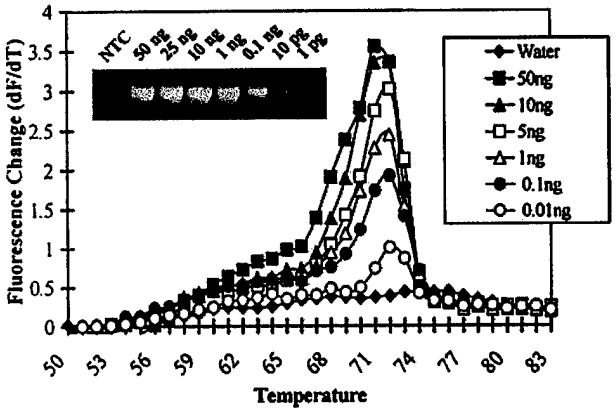

FIG. 3. Sensitivity of the hybridization probe CIP assay. The graph shows the melting peak analysis of the probe-PCR product hybrid as the change in fluorescence with the change in time (*dF/dT*) versus hybrid temperature at the various DNA concentrations. The 0.001-ng template reaction mixture was indistinguishable from the no-template control (water). NTC, no-template control.

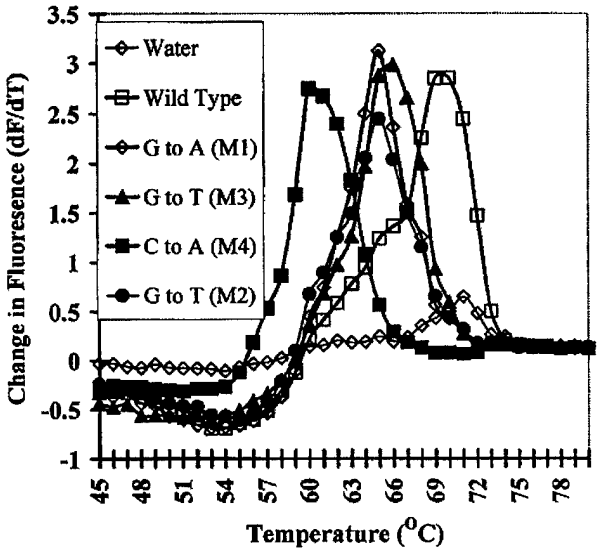

FIG. 4. FRET assay for Cp^r detection using purified template DNA of mutant and wild-type *Y. pestis*. Wild-type and mutant templates were used in the PCR amplification followed by melting peak analysis. The graph is of the change in fluorescence as a function of time (*dF/dT*) versus the temperature of the reaction products. The templates used in the various reactions are given in the key. Mutant designations in parentheses are as shown in Fig. 1A. All mutant templates produced a lower T_m than did the 100% match between probe 1 and the wild-type template. The template concentration was 10 μg/ml for all reactions.

primers LC3 and LC4 were designed to amplify a 261-bp region of *gyrA* that included all of the point mutations within the QRDR we had detected in our Cp^r mutant isolation (Fig. 1). As shown in Fig. 2, the FRET detection probe 1 was chosen to have the same sequence as wild-type *Y. pestis gyrA* and to encompass all four of the point mutations within the gene that resulted in Cp^r. FRET probe 2 was selected such that the 5′ end of the oligonucleotide containing the LightCycler-Red-640 label was positioned 1 bp away from the probe 1 3′ end (Fig. 2). The predicted melting temperatures (T_m) for probe 1 and probe 2 were 59.6 and 64.9°C, respectively. We reasoned that we would be able to detect the mutant genes through melting curve analysis by using a single probe, since probe 1 was based on the wild-type allele and included the region where all of the Cp^r point mutations occurred in *gyrA*.

We tested the ability of the hybridization probe assay to detect *Y. pestis gyrA* sequences using purified genomic DNA as a template. Melting peak analysis revealed that the change in fluorescence with the change in time versus temperature was also proportional to the amount of template DNA used in the reaction (Fig. 3). The ability to detect a change in fluorescence signal above background (no template controls) was proportional to the template concentration (Fig. 3). The hybridization probe assay was routinely able to detect *Y. pestis gyrA* sequences at concentrations of 10 pg of genomic DNA per reaction. Using a *Y. pestis* KIM 5 genome size of 4.4 Mbp (17), we calculate that our lower limit of detection of *gyrA* is approximately 2,100 genomic equivalents using purified DNA as a template in the reaction. We found that a single product of approximately 261 bp (Fig. 3, inset) was amplified in a template concentration-dependent manner similar to that seen on the FRET probe assay.

FRET probe detection of Cp^r *gyrA* DNA sequences. Using the FRET probe assay, we were able to differentiate *Y. pestis* KIM 5 *gyrA* mutants (Cp^r) from wild-type (Cp^s) organisms. As shown in Fig. 4, the melting peak temperature was dependent on the homology between probe 1 and the amplified PCR product. All of the mutant PCR products formed a less-stable hybrid with probe 1 than did the wild-type *gyrA* sequence. The largest difference in melting peak was seen with the cytosine-to-adenosine mutation (M4 in Fig. 1A), which was closest to the 3′ fluorescein label on probe 1. Interestingly, the most-stable probe1-mutant PCR product hybrid was formed with the guanine-to-thymine transversion mutant (Fig. 1A and 4). This mutation was located 1 bp 5′ to the mutation in the strain that formed the least-stable hybrid with probe 1 (M3 in Fig. 1A). However, the two mutations that were the most distal to the 3′ end of probe 1 (M1 and M2 in Fig. 1A) had an intermediate T_m. The mutant *gyrA*-probe1 T_m was easily differentiated from those generated with wild-type templates. The minimum T_m decrease compared to that for the wild-type sequence was with mutant M3, for which the T_m was consistently 4°C (Fig. 4). The maximum T_m shift compared to *Y. pestis* KIM 5 was greater than 11°C for the M4 mutant. Furthermore, we found the T_m of the melting peaks to be consistent between experiments. We performed five independent T_m determinations for all four mutants and the wild-type organism. The maximum standard deviation of the melting peak temperature obtained for each of these mutants from these experiments was 0.93°C. In all experiments the ΔT_m (defined as the T_m of the wild type − the T_m of the mutant) was also consistent and varied less than 0.5°C.

Colony assay for Cp^r *Y. pestis*. In order to test the utility of our FRET assay for identification of Cp^r *Y. pestis*, we performed the assay on crude lysates of bacteria. Initially, we used boiled lysates from a single colony suspended in 100 μl of water as the template in these reactions without any further growth of the cells. We found that we could identify the Cp^r

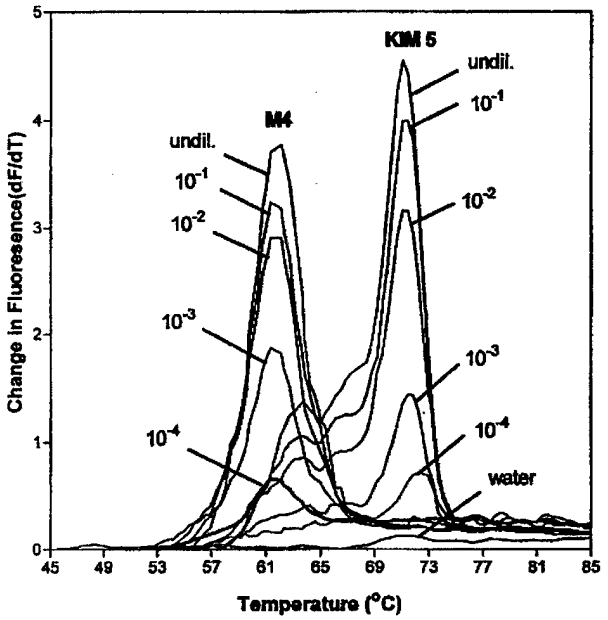

FIG. 5. Detection of Cp^r mutations in crude whole-cell lysates. Single colonies were grown in broth for 3 h at 30°C before being harvested. Shown is a melting peak analysis of various concentrations of wild-type *Y. pestis* or Cp^r mutant M4. Bacteria used in the reactions are given above each melting peak. Curves obtained with each dilution are labeled. The sample labeled "undil." represents the reaction obtained with undiluted bacterial suspensions. The 2-μl sample used in the FRET PCR labeled "undil." contained 4×10^4 CFU. Suspensions of bacteria were diluted before lysis by boiling to simulate different concentrations of organisms that might be obtained after the 3-h growth period. The curve labeled "water" represents the no-template control sample.

mutants in dilutions up to and including 1,000-fold (data not shown). The minimum number of CFU we could detect in our initial FRET PCRs was approximately 500. Since our goal was to develop our assay to cover as wide a range of template concentrations as possible, we tested the effect of growing the bacteria for a short time in liquid culture on the sensitivity of the assay. We found that growth for only 3 h in BHI broth allowed us to detect the presence of *Y. pestis gyrA* at dilutions up to 10,000-fold as shown in Fig. 5. This result represented an approximately 100-fold increase over reactions performed on suspended bacteria that were not allowed to replicate before analysis by the FRET assay. The FRET PCR generated from the 10^{-4} dilution of bacteria (Fig. 5) contained the equivalent of approximately 4 CFU of *Y. pestis*. Use of crude lysates as template DNA resulted in melting peak shifts for the Cp^r mutants that were similar to those seen with purified templates. The data shown in Fig. 5 are for the wild-type *gyrA* allele and mutant M4. Mutants M1, M2, and M3 all displayed melting peak temperature shifts similar to those shown in Fig. 4 (data not shown). Dilutions of cell suspensions greater than 10^{-4} resulted in melting peaks that could not be distinguished from those for negative controls. In order to compare melting peak analysis results with the amount of PCR product produced, we analyzed a 10-μl sample of the *Y. pestis* KIM 5 reaction products on agarose gels. We did not detect any *gyrA* PCR product in the reaction products produced with the 10^{-5} and 10^{-6} dilutions (data not shown).

DISCUSSION

Although Cp^r in *Y. pestis* is currently of no concern to public health officials, the organism is of major concern as a potential agent for biological warfare or bioterrorism (27). Given that CIP is a new-generation antibiotic under consideration for inclusion in a national antibiotic stockpile (9), a rapid method for the detection of Cp^r agents of bioterrorism is highly desirable. We have developed a simple and rapid PCR assay to detect Cp^r using FRET probes and the Roche Diagnostics LightCycler. The assay was able to detect four different point mutations within *Y. pestis* KIM 5 *gyrA* using a single probe pair with as few as approximately 2,100 copies of purified target sequence. Cp^r *Y. pestis* could also be detected in crude lysates prepared from fresh colonies isolated on petri plates at levels below 10 CFU per reaction. The difference in detection level between pure and crude templates is most likely due to the tendency of *Y. pestis* to grow as chains under culture conditions similar to those used here (L. E. Lindler, unpublished data). Using our assay, we were able to determine if the bacteria growing on solid medium were Cp^r within 4 h using melting peak analysis. Furthermore, the FRET assay was able to reliably identify Cp^r *Y. pestis* at CFU concentrations of 4 to 40,000 per reaction. This finding is particularly important if the method is to be useful in a clinical laboratory, since the assay will identify Cp^r bacteria over a wide range of template concentrations, thus reducing the possibility of false-negative reactions.

Given that FRET depends on the interaction of two fluorochromes in proximity with each other, it might be expected that the location of the base mismatch within probe 1 would strongly influence the melting peak. However, our results indicate that the position of the mismatch within probe 1 does not greatly influence this stability. This fact was suggested by our observation that mutant M4 and M3 produced the largest and smallest T_m values in our FRET assay and were located only 1 bp apart. The stability of these mutant templates was especially noteworthy, since M3 and M4 are located nearest the 3′ terminus of probe 1 and therefore might be expected to decrease the excitation of probe 2. If the position of the mismatch within the probe 1 sequence were a major factor in probe-template stability, then mutants M1 and M2 (Fig. 1A) might be expected to reduce the observed T_m to a lesser degree than mutant M3. However, the melting peaks of M1, M2, and M3 were very similar, further suggesting that the position of the mismatch did not influence probe 1 binding to the PCR product to any great degree. Taken together, our results suggest that the greatest influence on the observed melting peak using the FRET assay is due to the sequence of the base pair mismatch rather than the position of the mismatch within the donor probe.

Currently, the standard method of determining Cp^r is by agar diffusion tests. This method requires the isolation of the pathogen followed by an extra day of incubation with disks or strips. DNA-based methods for Cp^r detection, such as mismatch amplification mutation assay (MAMA) combined with DNA sequencing (32) and single-stranded confirmation poly-

morphism (23), have been developed. All of these methods require electrophoresis of reaction products to determine if a mutant allele of a cellular gyrase is encoded by the isolate. Also, both of the techniques above require equipment that does not easily lend itself to use in a clinical laboratory environment. Most recently, Wilson et al. (29) developed a 5′ nuclease assay to detect Cp^r in *Campylobacter jejuni* by PCR allelic discrimination (AD). These researchers were able to distinguish mutations at codon 86 by use of a pair of fluorescent probes and comparison of binding of those probes with either mutant or wild-type PCR products. Although this assay was shown to be sensitive to the femtogram level of template DNA, it has not been tested for the ability to distinguish Cp^r mutants from sensitive strains using crude whole-cell lysates as we have demonstrated here. Furthermore, we believe the FRET-based assay is more applicable because the use of a single pair of FRET probes allows the detection of four different linked point mutations in *gyrA*. In contrast, detection of the point mutations described here would require at least five different probes to be developed for an AD assay, assuming that one wild-type probe could be paired with a probe that binds to each individual point mutation in *gyrA*.

The mutations in *Y. pestis gyrA* all occurred at position 81 or 83 relative to the *E. coli* protein sequence. Although we did not determine the sequences of *gyrB* and *parC* for every Cp^r mutant we identified, our random sampling of these gene sequences suggests that they were most probably wild type in the mutants we characterized. Specifically, the observed MICs for all of the individual members of our four mutant classes were similar, and secondary alterations in *gyrB* and/or *parC* have been shown to result in increased levels of Cp^r (7, 11, 20). Taken together, our random DNA sequencing of known secondary mutation sites and our MIC data strongly suggest that the *Y. pestis* Cp^r strains we characterized did not encode mutations in the *gyrB* and/or *parC* QRDR.

Six different amino acid substitutions in *E. coli* GyrA have been identified in Cp^r strains following in vitro selection (4, 10, 22, 30). Among our 65 *Y. pestis* Cp^r mutants we found only two amino acid positions in GyrA that had been changed. The substitutions in *Y. pestis* GyrA were Gly-81 to Asp or Cys and Ser-83 to Ile or Arg. All of the amino acid substitutions we identified in *Y. pestis* GyrA have been found in other organisms (4, 15, 20, 23, 25, 30). Based on the results of our characterization of 65 *Y. pestis* Cp^r mutants, the most common site of mutation in GyrA is Gly-81. Other studies have indicated that Ser-83 is the most common hotspot for changes in *gyrA* that result in Cp^r (7, 11, 20, 21, 23, 30). The fact that Gly-81 appears to be a hotspot for mutation in *Y. pestis* may reflect a difference in the organism's DNA repair capabilities or a difference in the tertiary structure of GyrA. Alternatively, this finding may be due to the limited number of Cp^r isolates we characterized.

In summary, we have developed a pair of FRET probes that can easily detect four closely linked point mutations in *Y. pestis gyrA* by use of melting peak analysis. This FRET-based Cp^r detection method is sensitive, reproducible, and applicable over a wide range of template concentrations. In order for this assay to be useful in a clinical laboratory setting, it should be possible to perform the analysis as quickly after initial isolation of the organism as possible. We have demonstrated that this is possible by use of crude whole-cell lysates as templates in our reactions. Further testing of specificity is planned for future experiments. However, currently we envision that the FRET assay described here could be incorporated into a general DNA-based identification panel that would include *Y. pestis*-specific primers. Accordingly, it may be possible in the future to identify organisms and determine their antibiotic sensitivity profiles simultaneously. The development of our FRET-based Cp^r assay is a first step toward this goal.

ACKNOWLEDGMENTS

We thank Peter Schwartz for technical assistance. We also gratefully acknowledge the support of Erik Henchal.

This work is part of the U.S. Army Medical Research and Material Command Research Area Director IV sponsored programs.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Balfour, J. A., and D. Faulds.** 1993. Oral ciprofloxacin: a pharmacoeconomic evaluation of its use in the treatment of serious infections. Pharmacoeconomics **3**:398–421.
3. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277**:1453–1474.
4. **Cambau, E., F. Bordon, E. Collatz, and L. Gutmann.** 1993. Novel *gyrA* point mutation in a strain of *Escherichia coli* resistant to fluoroquinolones but not to nalidixic acid. Antimicrob. Agents Chemother. **37**:1247–1252.
5. **Caplin, B. E., R. P. Rasmussen, P. S. Bernard, and C. T. Wittwer.** 1999. LightCycler hybridization probes: the most direct way to monitor PCR amplification for quantitation and mutation detection. Biochemica **1**:5–9.
6. **Chu, Y. W., E. T. Houang, and A. F. Cheng.** 1998. Novel combination of mutations in the DNA gyrase and topoisomerase IV genes in laboratory-grown fluoroquinolone-resistant *Shigella flexneri* mutants. Antimicrob. Agents Chemother. **42**:3051–3052.
7. **Deguchi, T., A. Fukuoka, M. Yasuda, M. Nakano, S. Ozeki, E. Kanematsu, Y. Nishino, S. Ishihara, Y. Ban, and Y. Kawada.** 1997. Alterations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV in quinolone-resistant clinical isolates of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **41**:699–701.
8. **Gensberg, K., Y. F. Jin, and L. J. Piddock.** 1995. A novel *gyrB* mutation in a fluoroquinolone-resistant clinical isolate of *Salmonella typhimurium*. FEMS Microbiol. Lett. **132**:57–60. (Erratum, **137**:293, 1996.)
9. **Grabenstein, J., K. Downs, and D. Dotson.** 2000. Extraordinary infections: a focus on bioterrorism. J. Am. Pharm. Assoc. (Washington, D.C.) **40**:S36–S37.
10. **Hallett, P., and A. Maxwell.** 1991. Novel quinolone resistance mutations of the *Escherichia coli* DNA gyrase A protein: enzymatic analysis of the mutant proteins. Antimicrob. Agents Chemother. **35**:335–340.
11. **Heisig, P.** 1996. Genetic evidence for a role of *parC* mutations in development of high-level fluoroquinolone resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **40**:879–885.
12. **Heisig, P.** 1993. High-level fluoroquinolone resistance in a *Salmonella typhimurium* isolate due to alterations in both *gyrA* and *gyrB* genes. J. Antimicrob. Chemother. **32**:367–377.
13. **Jack, D. B.** 1996. Drug-resistant bacteria: responding to the infectious disease crisis. Mol. Med Today **2**:499–502.
14. **Jones, R. N., and M. A. Pfaller.** 1998. Bacterial resistance: a worldwide problem. Diagn. Microbiol. Infect. Dis. **31**:379–388.
15. **Korten, V., W. M. Huang, and B. E. Murray.** 1994. Analysis by PCR and direct DNA sequencing of *gyrA* mutations associated with fluoroquinolone resistance in *Enterococcus faecalis*. Antimicrob. Agents Chemother. **38**:2091–2094.
16. **Koshland, D. E., Jr.** 1994. The biological warfare of the future. Science **264**:327.
17. **Lucier, T. S., and R. R. Brubaker.** 1992. Determination of genome size, macrorestriction pattern polymorphism, and nonpigmentation-specific deletion in *Yersinia pestis* by pulsed-field gel electrophoresis. J. Bacteriol. **174**:2078–2086.
18. **Michel, M., and L. Gutmann.** 1997. Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci: therapeutic realities and possibilities. Lancet **349**:1901–1906.
19. **Moss, A. R., D. Alland, E. Telzak, D. Hewlett, Jr., V. Sharp, P. Chiliade, V. LaBombardi, D. Kabus, B. Hanna, L. Palumbo, K. Brudney, A. Weltman, K. Stoeckle, K. Chirgwin, M. Simberkoff, S. Moghazeh, W. Eisner, M. Lutfey, and B. Kreiswirth.** 1997. A city-wide outbreak of a multiple-drug-resistant

strain of *Mycobacterium tuberculosis* in New York. Int. J. Tuberc. Lung Dis. **1**:115–121.
20. **Mouneimne, H., J. Robert, V. Jarlier, and E. Cambau.** 1999. Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43**:62–66.
21. **Ruiz, J., F. Marco, P. Goni, F. Gallardo, J. Mensa, A. Trilla, T. Jimenez de Anta, and J. Vila.** 1995. High frequency of mutations at codon 83 of the *gyrA* gene of quinolone-resistant clinical isolates of *Escherichia coli*. J. Antimicrob. Chemother. **36**:737–738.
22. **Swanberg, S. L., and J. C. Wang.** 1987. Cloning and sequencing of the *Escherichia coli gyrA* gene coding for the A subunit of DNA gyrase. J. Mol. Biol. **197**:729–736.
23. **Takenouchi, T., E. Sakagawa, and M. Sugawara.** 1999. Detection of *gyrA* mutations among 335 *Pseudomonas aeruginosa* strains isolated in Japan and their susceptibilities to fluoroquinolones. Antimicrob. Agents Chemother. **43**:406–409.
24. **Thomson, C. J.** 1999. The global epidemiology of resistance to ciprofloxacin and the changing nature of antibiotic resistance: a 10 year perspective. J. Antimicrob. Chemother. **43**(Suppl. A):31–40.
25. **Truong, Q. C., J. C. Nguyen Van, D. Shlaes, L. Gutmann, and N. J. Moreau.** 1997. A novel, double mutation in DNA gyrase A of *Escherichia coli* conferring resistance to quinolone antibiotics. Antimicrob. Agents Chemother. **41**:85–90.
26. **Weigel, L. M., C. D. Steward, and F. C. Tenover.** 1998. *gyrA* mutations associated with fluoroquinolone resistance in eight species of *Enterobacteriaceae*. Antimicrob. Agents Chemother. **42**:2661–2667.
27. **Weller, R. E., C. W. Lyu, C. Wolters, and R. M. Atlas.** 1999. Universities and the biological and toxin weapons convention. ASM News **65**:403–409.
28. **Wiedemann, B., and P. Heisig.** 1994. Mechanisms of quinolone resistance. Infection **22**:S73–S79.
29. **Wilson, D. L., S. R. Abner, T. C. Newman, L. S. Mansfield, and J. E. Linz.** 2000. Identification of ciprofloxacin-resistant *Campylobacter jejuni* by use of a fluorogenic PCR assay. J. Clin. Microbiol. **38**:3971–3978.
30. **Yoshida, H., M. Bogaki, M. Nakamura, and S. Nakamura.** 1990. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob. Agents Chemother. **34**:1271–1272.
31. **Yoshida, H., M. Bogaki, M. Nakamura, L. M. Yamanaka, and S. Nakamura.** 1991. Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. Antimicrob. Agents Chemother. **35**:1647–1650.
32. **Zirnstein, G., Y. Li, B. Swaminathan, and F. Angulo.** 1999. Ciprofloxacin resistance in *Campylobacter jejuni* isolates: detection of *gyrA* resistance mutations by mismatch amplification mutation assay PCR and DNA sequence analysis. J. Clin. Microbiol. **37**:3276–3280.